REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	
2. REPORT TYPE	Final
3. DATES COVERED *(From - To)*	
4. TITLE AND SUBTITLE	An Evaluation of an Inline Sensor for Detection of Microbial Activity
5a. CONTRACT NUMBER	
5b. GRANT NUMBER	
5c. PROGRAM ELEMENT NUMBER	0602236N
5d. PROJECT NUMBER	
5e. TASK NUMBER	
5f. WORK UNIT NUMBER	73M13403
6. AUTHOR(S)	Lee, Jason S., Ray, Richard I., Little, Brenda J.
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	Naval Research Laboratory Oceanography Division Stennis Space Center, MS 39529-5004
8. PERFORMING ORGANIZATION REPORT NUMBER	NRL/PP/7303--04-0005
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	Office of Naval Research 800 N. Quincy St. Arlington, VA 22217-5660
10. SPONSOR/MONITOR'S ACRONYM(S)	ONR
11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT	Approved for public release, distribution is unlimited.
13. SUPPLEMENTARY NOTES	

14. ABSTRACT

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15. SUBJECT TERMS

biofilm, polarization, electrode

16. SECURITY CLASSIFICATION OF: a. REPORT	b. ABSTRACT	c. THIS PAGE	17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
Unclassified	Unclassified	Unclassified	UL	17	Jason S. Lee
					19b. TELEPHONE NUMBER *(Include area code)* 228-688-4494

Paper
05497

CORROSION2005

AN EVALUATION OF AN INLINE SENSOR FOR DETECTION OF MICROBIAL ACTIVITY

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ABSTRACT

Laboratory experiments were designed to determine the influence of polarization (±175 mV vs. saturated calomel electrode) in natural fresh water and in dilute microbiological media (1:100 Luria-Bertani broth) on biofilm formation on 316L stainless steel. Biofilms formed on all polarized and unpolarized surfaces within 120 hours. Variability among the surfaces was detected with environmental scanning electron microscopy. Polarization influenced microbial settlement in both media. Biofilm formation on polarized surfaces may not represent natural biofilms formed in the absence of polarization.

INTRODUCTION

Physical separation of anodes and cathodes and subsequent measurement of some electrochemical parameter have been used to detect biofilm formation and to evaluate the electrochemical impact of biofilms.[1-3] In some cases polarization has been used.[1,2] Angell *et al.*[1] used a concentric ring 304 stainless steel electrode to demonstrate that a consortium of sulfate-reducing bacteria and a *Vibrio* sp. maintained a galvanic current between the anode and cathode. In their electrode design, pitting was induced by passage of a 11 $\mu A\ cm^{-2}$ current density to a small (0.031 cm^2) anode. The anode was concentric to, and separated from, the cathode (4.87 cm^2) by a Teflon®

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(polytetrafluoroethylene) spacer. Current was applied for seventy-two hours either during or after microbial colonization. Once the applied current was removed the resultant galvanic current flowing between the anode and the cathode was monitored by a zero resistance ammeter. They found that a current was maintained in the presence of a microbial consortium. No current was measured in a sterile control. Licina and Nekoksa[2] developed a probe consisting of ten 316 stainless steel concentric rings separated by epoxy. A potential (which varies according to experimental conditions) is imposed for 1 hour each day between the electrodes so that the electrodes are alternately anodes and cathodes. The metal discs are polarized to produce an environment "conducive to biofilm formation." The applied current, required to achieve a pre-set potential between electrodes, remains stable until a biofilm forms. Once a biofilm is established, current increases consistent with a decrease in the polarization resistance. The generated current, current that continues to flow between the electrodes after the external polarization has been removed, is another indication of biofilm development. In the absence of a biofilm the generated current is expected to be zero. In the presence of a biofilm some current is expected to flow.

While the Angell *et al.*[1] and the Licina and Nekoksa[2] probes are similar in that both rely on separation of anode and cathode regions for detection of microbial presence, there are significant differences. The concentric ring electrode[1] provides a technique by which microbiologically influenced corrosion (MIC) can be studied and is not intended to represent any natural situation. The commercial probe[2] is intended to provide information about an operating system (such as a flowing cooling water piping) that can be used to make decisions about cleaning or treatment. The probe designs also differ in the motivation for polarization. Angell *et al.*[1] induced pitting in the anode. Franklin *et al.*[4] and Little *et al.*[5] were among the first to demonstrate that bacteria are attracted to anodic sites. The spatial relationship between corroding ferrous materials and bacteria has been demonstrated using several techniques and electrolytes.[6-8] There is general agreement among investigators that bacteria are attracted to anodic sites on ferrous materials. Polarization of the multiple ring probe does not cause localized corrosion and is designed to "encourage biofilm formation." Literature on the impact of polarization of biofilm development is confusing and difficult to compare because of differing experimental conditions (laboratory vs. field) in addition to differing metals, microorganisms, electrolytes and techniques to evaluate constituents within biofilms. The experiments described in the paper were designed to evaluate biofilm development in the presence of polarizations typical of a biofilm probe.

METHODS AND MATERIALS

The impact of polarization on attachment of bacteria was evaluated using two electrode types: 1) a commercially available probe (Figure 1a) consisting of 10 concentric rings of 316L stainless steel separated by epoxy rings and 2) a flat (2 cm^2) 316L stainless steel electrode embedded in epoxy (Figure 1b). The commercially available biofilm sensor was used according to manufacturer's instructions. A potential of 350 mV was imposed between alternating rings for 1 hour per day, producing 2 sets of polarized rings (one positive and one negative) using a standard three-electrode potentiostat. The working electrode connection was attached to the 'positive' set while the counter and reference electrode connections were connected to the 'negative' set. The electrodes were examined with a Wild Typ 376788 light microscope (500X max) every 24 hours to evaluate bacterial attachment. At the first indication of microbial settlement, the electrodes were rinsed in distilled water and examined with environmental scanning electron microscopy.[9] Bacterial attachment on the commercial probe was also evaluated in the absence of polarization at open-circuit potential (E_{corr}).

Bacterial attachment to the 316L stainless steel electrodes embedded in epoxy was evaluated under three different polarization conditions: 1) +175 mV (in reference to the established E_{corr} value) for 1 hour per day, 2) polarized -175 mV (vs. E_{corr}) and 3) no polarization. ±175 mV polarization was selected to represent conditions similar to those of the commercial probe *i.e.*, positive and negative electrodes with a 350 mV applied potential difference. A three-electrode potentiostat was employed, but in contrast to polarization of the commercial probe, a platinum/niobium mesh and a saturated calomel electrode (SCE) were used as counter and reference electrodes, respectively. Similarly, the electrodes were exposed until bacterial attachment could be detected, as previously described.

Microbial settlement on electrodes was evaluated in two media: 1:100 Luria-Bertani (LB) in distilled water inoculated with *Shewanella* sp. >10^{10} cells ml^{-1} and natural pond water (Sabine River at Galveston, TX) containing a diverse microflora >10^{10} cells ml^{-1}. Conductivity, NaCl concentration and total organic carbon for both media are presented in Table 1.

Table 1. Chemistry of media.

Medium	Conductivity ($\mu S/cm^2$)	[NaCl] (ppm)	Total Organic Carbon (mg/L)
1:100 Luria-Bertani in dH_2O	245	100	>60
Natural Pond Water	655	300	15

RESULTS

For the case of the embedded 316L electrode exposed to LB media, the current resulting from the application of +175 mV (vs. E_{corr}) for 2 one-hour intervals (1 at the time of immersion and a second after 24 hours of exposure) is presented in Figure 2a on log scale as a function of time of polarization. One observation is that current was less scattered after 24 hours and wass entirely positive (anodic). The current after 24 hours also decreased by about half from the first polarization. After 24 hours the surface of the electrode wass covered with debris and *Shewanella* cells were uniformly distributed on the surface (Figure 2b-d). Larger patches of extra-polymeric substances (EPS) were also observed. In the case of the electrode with an applied voltage of –175 mV, Figure 3a indicates that the current (linear scale) was less scattered after 24 hours. In contrast to the positively polarized electrode, the current alternates between positive (anodic) and negative (cathodic). The surface was uniformly covered with *Shewanella* cells but did not have large patches of debris and EPS (Figure 3b-d). As shown in Figure 4 the unpolarized electrode surface was also uniformly covered with cells and like the positively polarized electrode, has large patches of EPS.

A potential of 350 mV was applied between the 2 sets of alternating electrode rings of the commercial probe during the same period as the embedded electrodes. The applied current (Figure 5a) indicated that current decreased after 24 hours. *Shewanella* cells were uniformly distributed on the positive and negative electrode rings (not shown) while no large patches of debris or EPS were observed (Figure 5b). The commercial probe that was not polarized for the 24-hour period had a different surface appearance. Near the air/water interface (Figure 6a), a large patch of biofilm was centered on the intersection between the polymeric separator and the 316L rings. The portion of the probe, which was fully submersed, had an even distribution of biofilm patches on both the 316L rings and the polymeric separators (Figure 6b).

Exposure of the embedded electrode to the natural pond water containing a natural bacterial consortium required 5 one-hour polarizations over a 120-hour period before bacteria settlement could be detected on the electrode surface. Application of +175 mV (vs. E_{corr}) resulted in current which became more erratic with time (Figure 7a), in contrast to the laboratory media exposure which indicated less scatter with time (Figure 2a). The current dropped an order of magnitude between the first and second 1-hr polarizations. After 120 hours, bacteria were evenly distributed on the surface on the +175 electrode (Figure 7b-c). No large patches of debris or EPS were seen and algae were also detected on the surface. The applied current for the 5-one hour polarizations of –175 mV (vs. E_{corr}) is presented in Figure 8a. Positive and negative current was observed, as in the laboratory media exposure, with a decrease in scatter as exposure time increases. Figure 8b-c indicates the same electrode surface appearance as observed on the positively polarized electrode. The surface of the unpolarized electro was covered with large amounts algae and a patchy biofilm was observed after 120 hours (Figure 9).

The applied current from 350 mV polarization between electrode rings of the commercial probe initially increased after 24 hours of exposure, then decreased with further exposure (Figure 10). In the cases of both the positive (Figure 11) and negative (Figure 12) electrode rings, bacteria were uniformly distributed on both electrode surfaces, as well as on the dividing polymeric separators. However, patches of biofilm were more prevalent on the polymeric separators and at the interfaces with the 316L rings (Figures 11a and 12a). In contrast, the unpolarized commercial electrode had patches of biofilm evenly distributed on both metal and polymeric surfaces (Figure 13). One general observation with the commercial probe was that bacteria attached first at the intersection between the polymeric separators and the 316L rings regardless of the presence of polarization or its polarity. Cleaning microorganisms from this location required sonication.

DISCUSSION

After decades of investigating cell/surface interactions there are many observations, but few conclusions about the factors controlling attachment. Cellular factors known to influence attachment include particular proteins, extracellular polymers, appendages such as flagella and fimbriae, degree of cell surface hydrophobicity and electrostatic charge, mobility, cell size and the nutritional and physiological status of the cell. Physiochemical aspects of the substratum and the liquid phase that influence attachment include temperature, surface free energy, hydrophobicity, polarization electrostatic charge, polarization, ionic strength and the presence of metabolizable carbon. Each of these cellular and substratum influences can vary with environment. The tremendous diversity and flexibility in the mechanisms and strategies with which microorganisms attach to solid surfaces confounds conclusions.

Many bacteria are negatively charged and are attracted to positive charge.[10, 11] However the literature on the subject is confusing. Nekoksa and Gutherman[12] and Guezennec[13] showed that more marine bacteria settled on cathodically protected metals than on unpolarized metals. Gomez de Saravia *et al.*[14] and Videla *et al.*[15] demonstrated that cathodic polarization in a seawater medium increased numbers of sulfate-reducing bacteria and decreased numbers of aerobic bacteria on carbon steel relative to unpolarized surfaces. De Sanchez and Schiffrin[16] demonstrated chemotaxis of a marine bacterium induced by transition metal ion concentration gradients from corroding copper and titanium. van Schie and Fletcher[10] demonstrated that the presence of Fe^{+3} on the substratum significantly increased attached cells.

In the experiments described in this paper, polarization influenced the distribution of bacteria and algae on the surface of 316L stainless steel. Biofilms in artificial and natural water (1:100 Luria-Bertani broth and Sabine River at Galveston, TX, respectively) settled preferentially on unpolarized surfaces. The motivation for polarizing the commercial and laboratory probes was two-fold: 1) encourage biofilm formation and 2) evaluate applied current. Generated current between anodic and cathodic sites after polarization was not recorded in our experiments. Our study only examined the effect of potentiostatically applied current on bacterial settlement. As previously stated, the applied current is employed to achieve a pre-set potential between electrodes. In contrast, the generated current is the current that continues to flow between the electrodes after the external polarization has been removed.

In this study, applied current in general decreased in magnitude and scatter after each successive polarization in all polarized exposure conditions. These results can be attributed to a decrease in the passive current density with each subsequent polarization. When polarized in the noble direction (+175 mV) the resulting 316L electrode potential is in the passive region of the anodic polarization curve of this stainless steel well below the pitting potential for the low concentration of chloride ion.[17] Holding the potential in the passive regions causes the Cr rich passive film to grow, thereby decreasing the passive current density when polarization is terminated. Each subsequent polarization causes a thickening of the passive film and a further decrease in passive current. Cathodic polarization (-175 mV) of the 316 electrodes also produced lower applied (both positive and negative) current magnitude during each subsequent polarization attributable to cathodic protection of the electrode. Decrease in current magnitude scatter in both anodic and cathodic polarization may also be attributed to healing of submicron scale pits in the passive surface. Initially, surfaces, even polished surfaces, contain many submicron scale pits. Polarization causes the healing of these pits in the passive layer. Further controlled experiments are required to determine the effect of bacterial settlement on applied current.

The applied current behavior (*i.e.* decrease) observed in this study was also observed by Licina[18] using the same commercial probe in a variety of industrial settings. Licina saw a decrease in applied current, or stabilization, after several days exposure, followed by an increase in applied current. Licina attributes the increase in current to the formation of an intact biofilm and to a subsequent increase in microbial activity. The lack of applied current increase in our study may be due patchy biofilm surface coverage. Since the exposure was terminated at the first observation of bacterial settlement, a fully mature biofilm covering the majority of the electrode surface was never achieved. It should also be noted that the increase in applied current seen by Licina may in fact not be attributed to an attached mature biofilm at the electrode surface. Experiments involving biological fuel cells by Rabey *et al.*[19] suggest that two methods of electron transfer from cells to electrode surfaces. Surface bound cells transfer electrons to the anodic electrode through cell membrane directly. Soluble redox mediators produced by cells facilitate discharge of an electron to the electrode even when the cell was not attached to the surface. Figure 14 indicates an example of this mechanism as explained by Reimers *et al.*[20] An increase in applied current could result from a biofilm on the electrode surface or from suspended cells.

CONCLUSIONS

Experiments described in this paper have demonstrated:

- Polarization influences bacterial settlement.
- Polarization is not required for bacterial settlement.
- Bacterial settlement on polarized surfaces may not represent natural biofilms.
- Bacteria may not dominate biofilms in natural environments. Instead, filamentous algae, diatoms or fungi could potentially predominate.
- Production of soluble redox mediators by planktonic bacteria suggests that attributing an increase in applied current solely to biofilm formation may be inaccurate.

ACKNOWLEDGEMENTS

This work was supported by the Office of Naval Research Program Element 0602236N and from funding provided by DuPont Inc. work unit number 73M13403. NRL publication number NRL/PP/7303-04-5.

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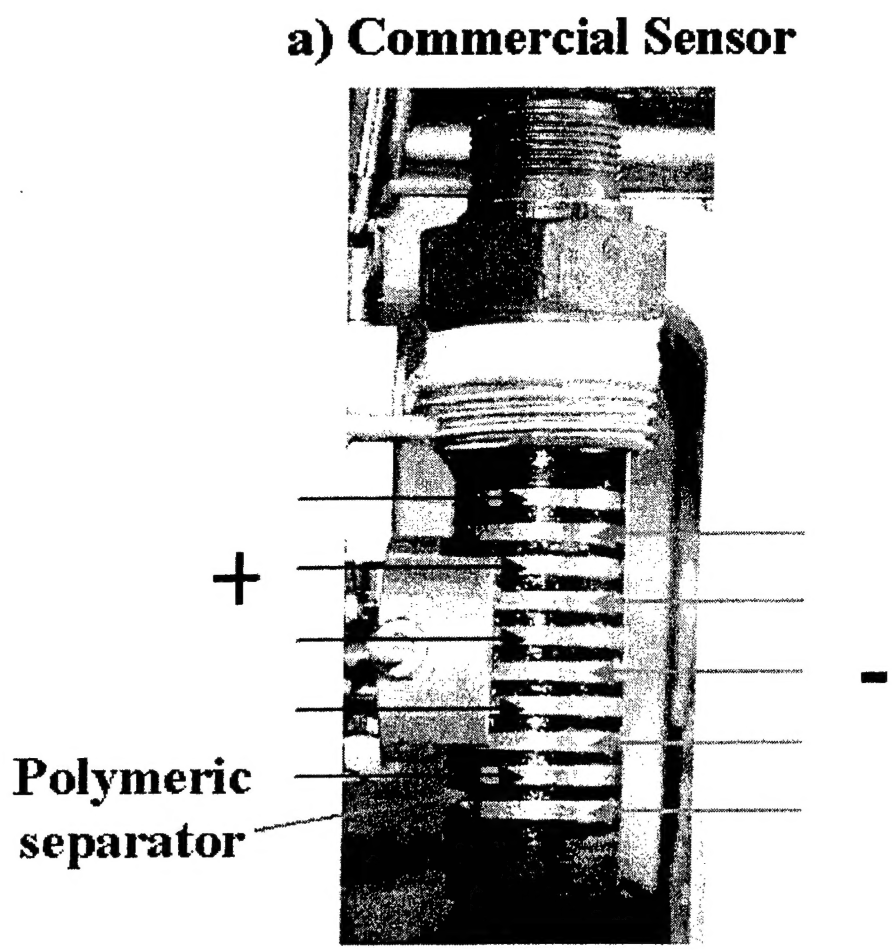

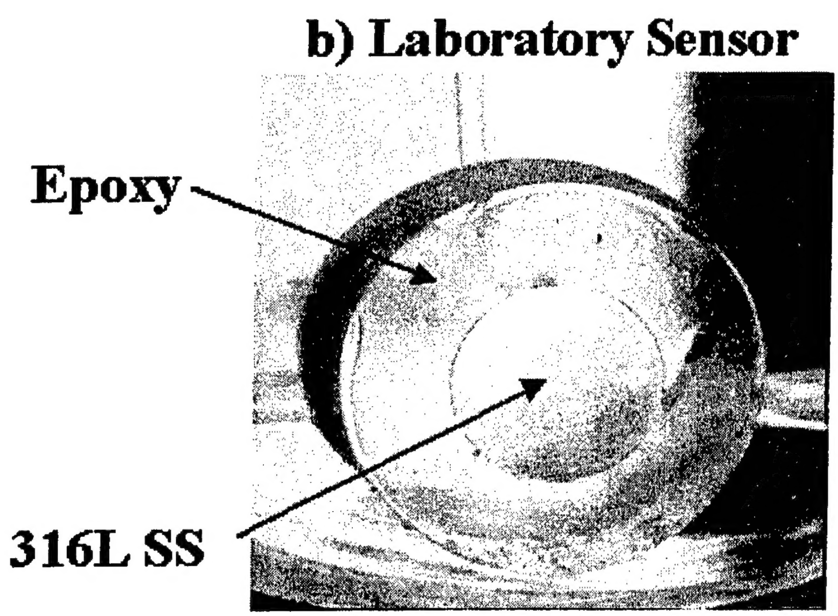

Figure 1. a) Commercial sensor with alternating positively and negatively polarized 316L rings separated by polymeric insulating rings. b) Laboratory sensor consisting of 2 cm^2 area 316L electrode embedded in epoxy.

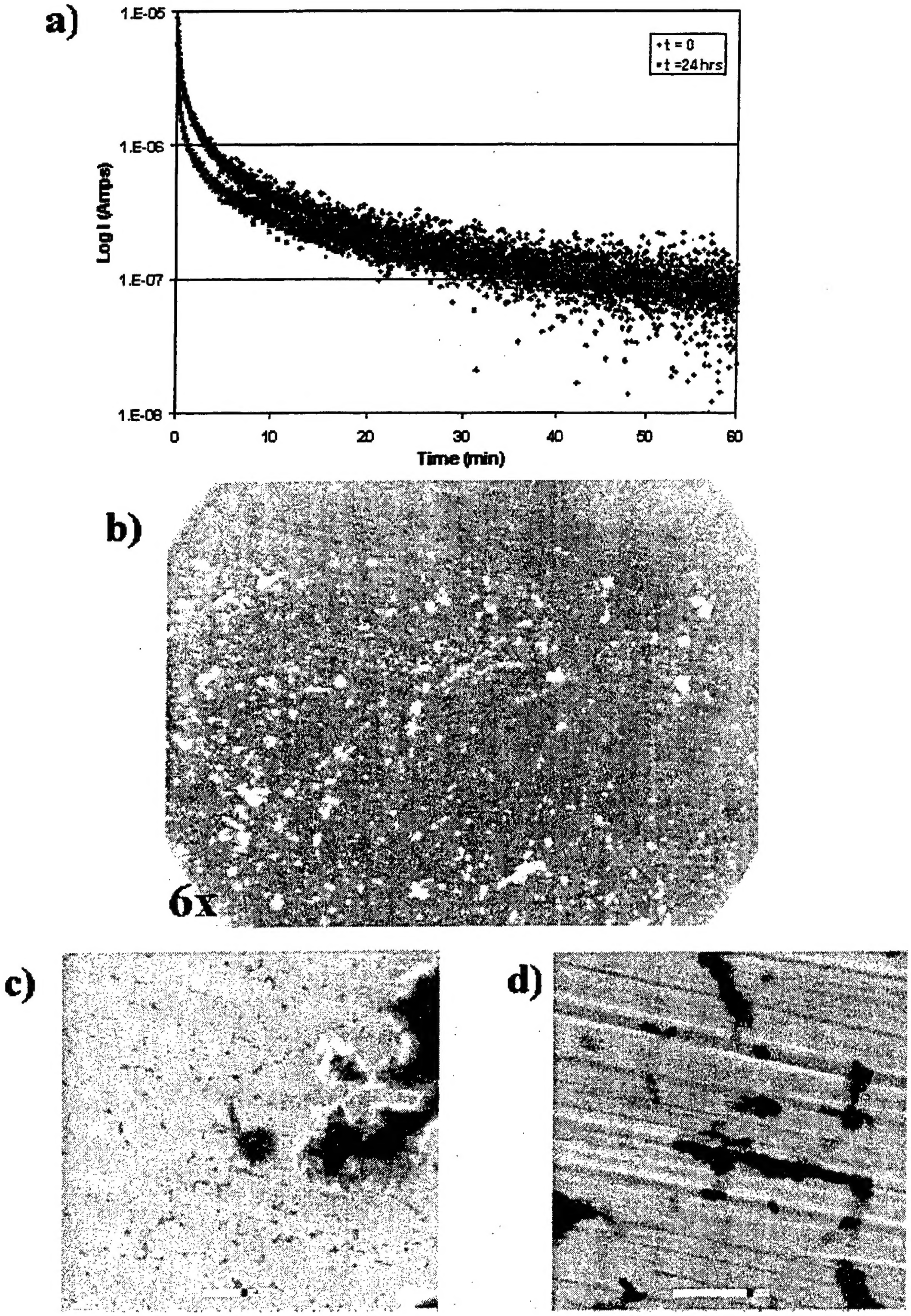

Figure 2. Laboratory electrode exposed to 1:100 Luria-Bertani in dH_2O for 24 hrs with anodic polarization. a) Applied current responses (log scale) for potentiostatic holds (1 hr duration) at +175 mV vs. E_{corr} at exposure times of 0 and 24 hrs. b) Optical image of electrode surface after 24 hrs exposure. c&d) Micrographs of electrode surface after 24 hr exposure.

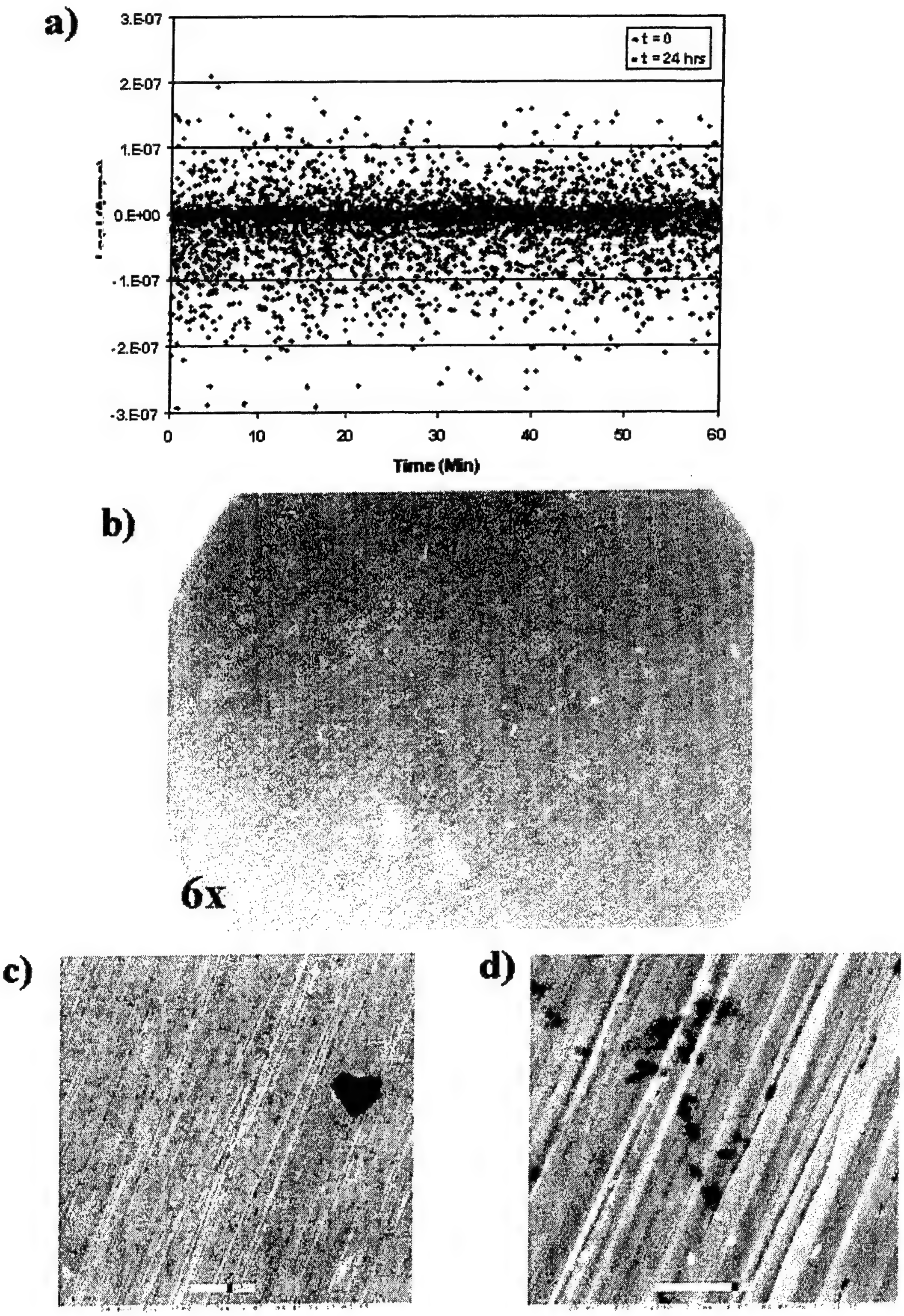

Figure 3. Laboratory electrode exposed to 1:100 Luria-Bertani in dH_2O for 24 hrs with cathodic polarization. a) Applied current responses (linear scale) for potentiostatic holds (1 hr duration) at -175 mV vs. E_{corr} at exposure times of 0 and 24 hrs. b) Optical image of electrode surface after 24 hrs exposure. c&d) Micrographs of electrode surface after 24 hr exposure.

a)

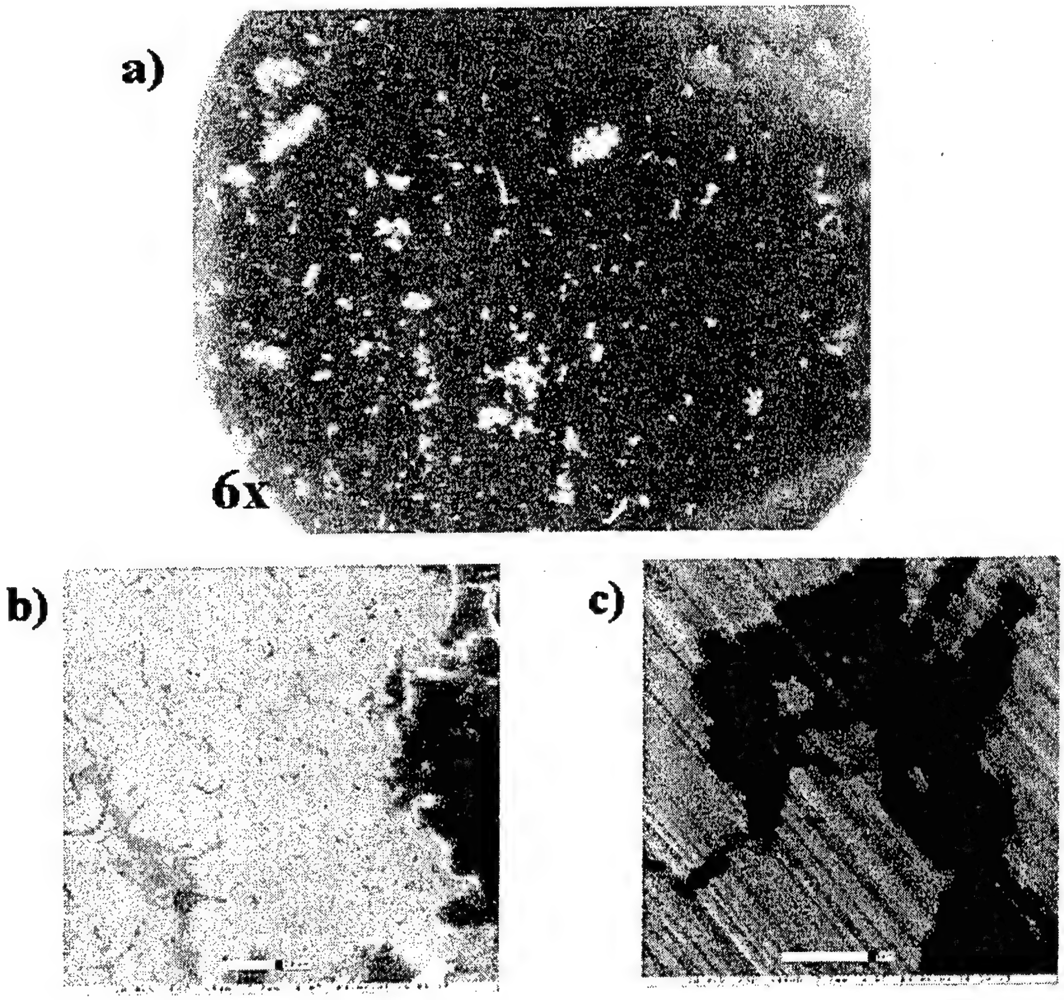

Figure 4. Laboratory electrode exposed to 1:100 Luria-Bertani in dH_2O for 24 hrs with no polarization. a) Optical image of electrode surface after 24 hr exposure. b&c) Micrographs of electrode surface after 24 hr exposure.

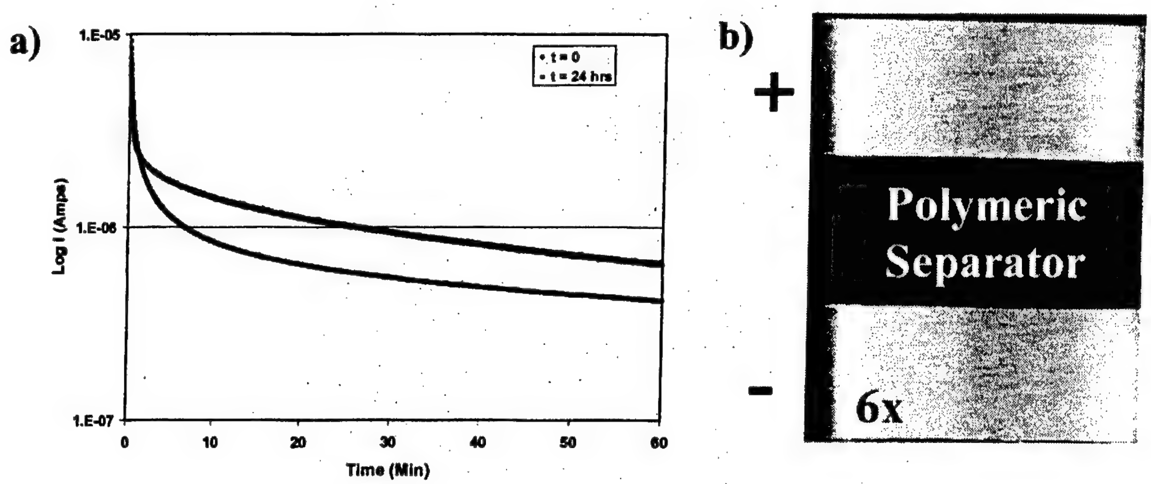

Figure 5. Commercial probe exposed to 1:100 Luria-Bertani in dH_2O for 24 hrs with an applied potential of 350 mV (1 hr duration) between the two sets of alternating electrode rings. a) Applied current responses (log scale) for 350 mV potential holds at exposure times of 0 and 24 hrs. b) Optical image of positive and negative electrode surfaces after 24 hr exposure. No evidence of biofilm formation after 24 hrs. No SEM images available.

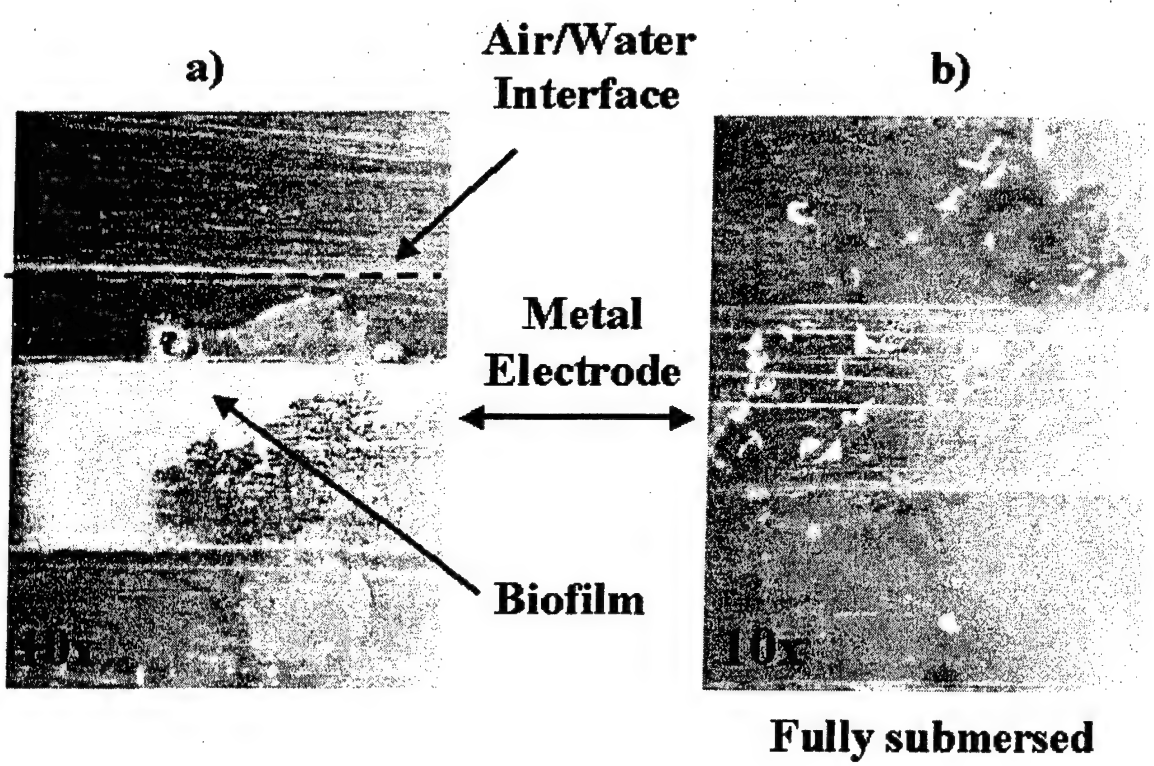

Figure 6. Commercial probe exposed to 1:100 Luria-Bertani in dH_2O for 24 hrs with no applied potential. a) Optical image of electrode surface near water line after 24 hr exposure. b) Optical image of a fully submerged electrode surface after 24 hr exposure.

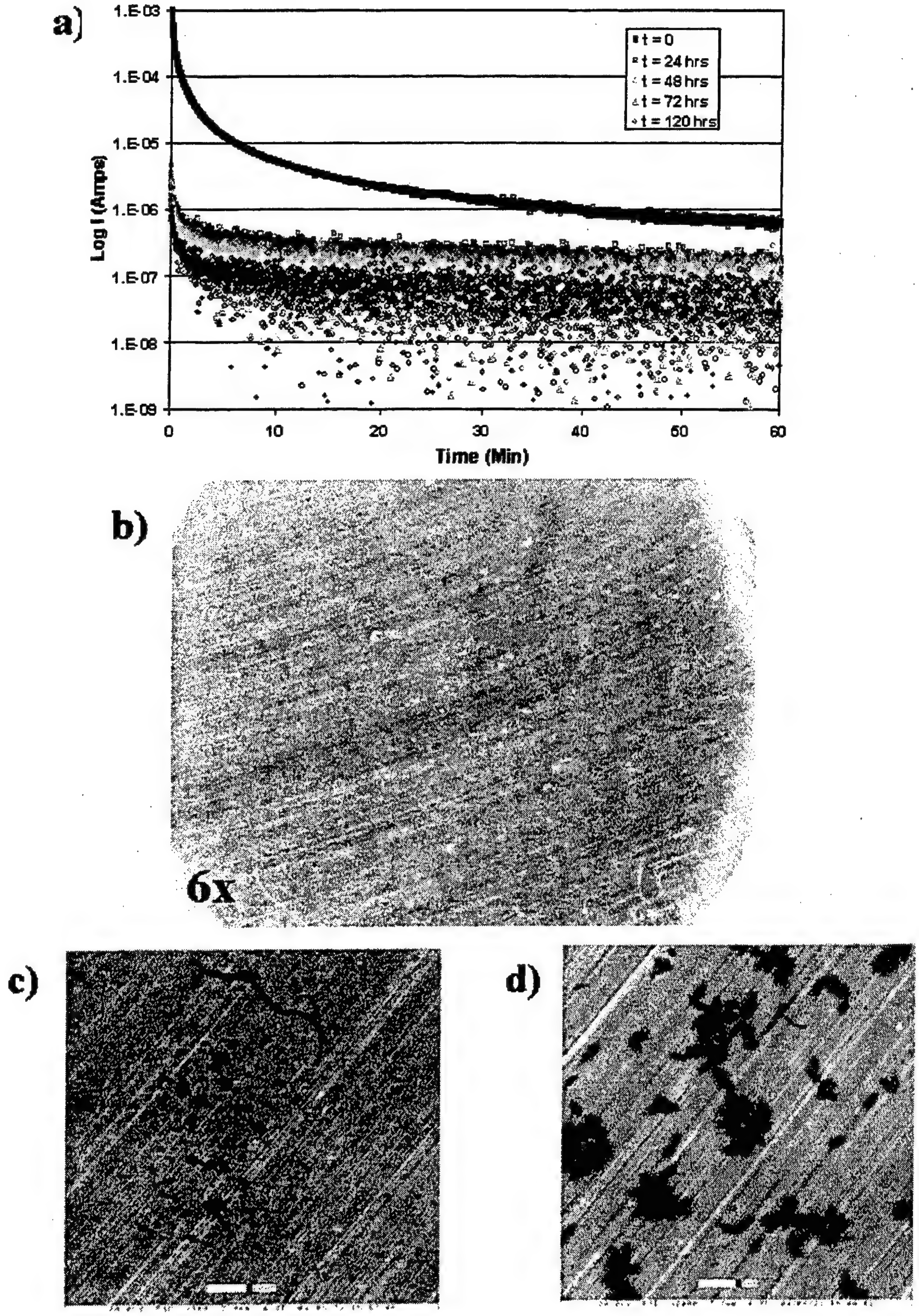

Figure 7. Laboratory electrode exposed to natural pond water for 120 hrs with anodic polarization every 24 hrs. a) Applied current responses (log scale) for potentiostatic holds (1 hr duration) at +175 mV vs. E_{corr} every 24 hrs. b) Optical image of electrode surface after 120 hr exposure. c&d) Micrographs of electrode surface after 120 hr exposure.

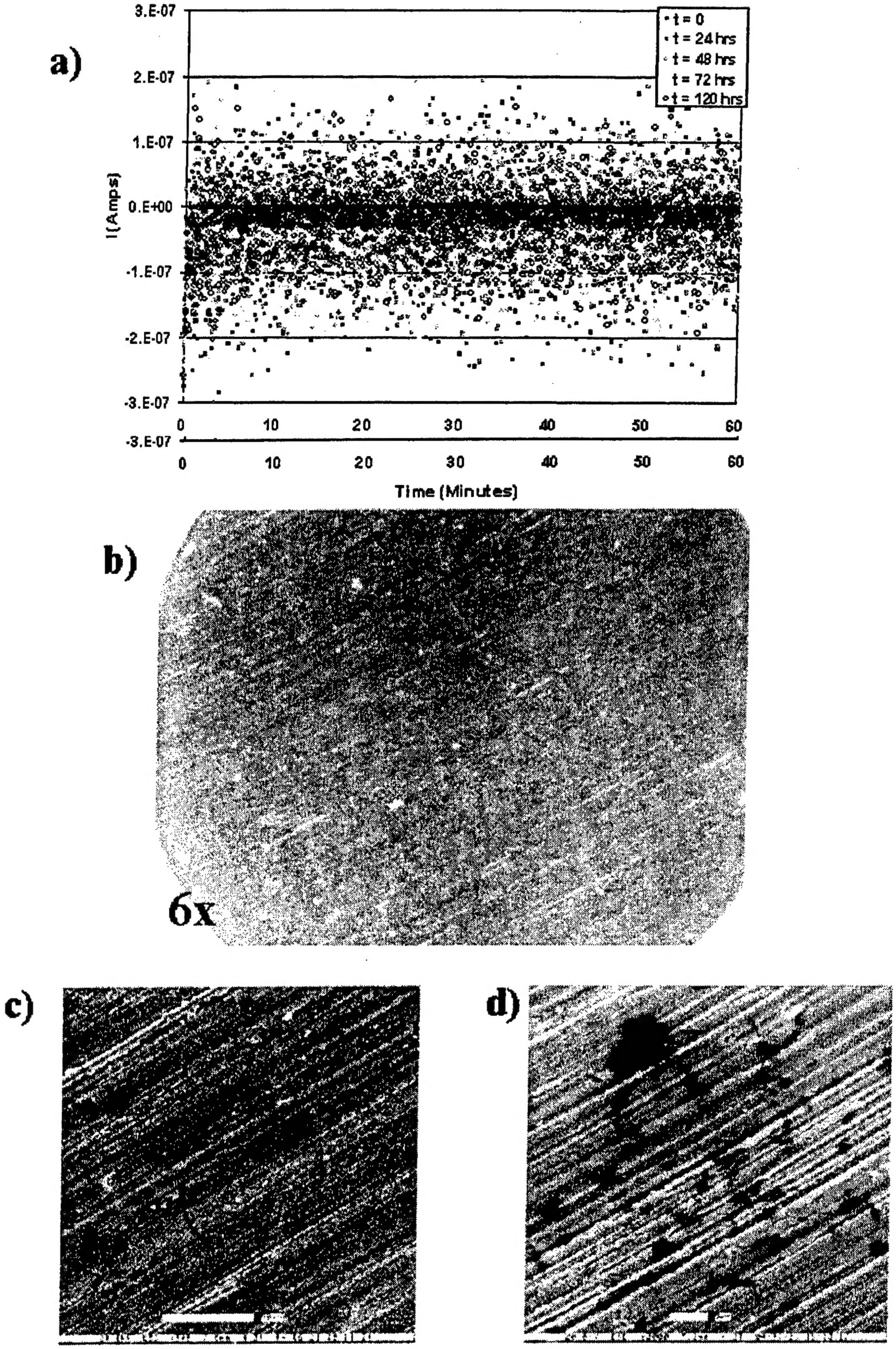

Figure 8. Laboratory electrode exposed to natural pond water for 120 hrs with cathodic polarization every 24 hrs. a) Applied current responses (linear scale) for potentiostatic holds (1 hr duration) at -175 mV vs. E_{corr} every 24 hrs. b) Optical image of electrode surface after 120 hr exposure. c&d) Micrographs of electrode surface after 120 hr exposure.

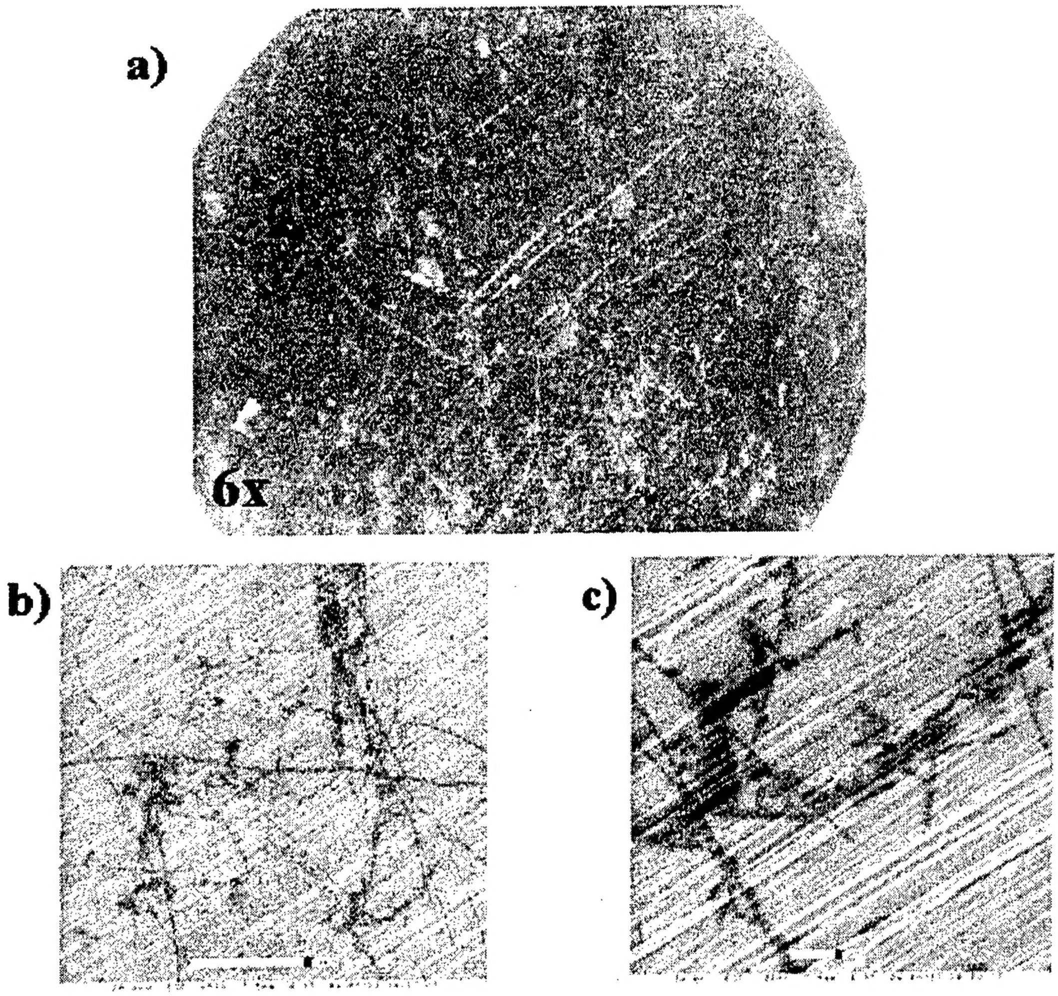

Figure 9. Laboratory electrode exposed to natural pond water for 120 hrs with no polarization. a) Optical image of electrode surface after 120 hr exposure. b&c) Micrographs of electrode surface after 120 hr exposure.

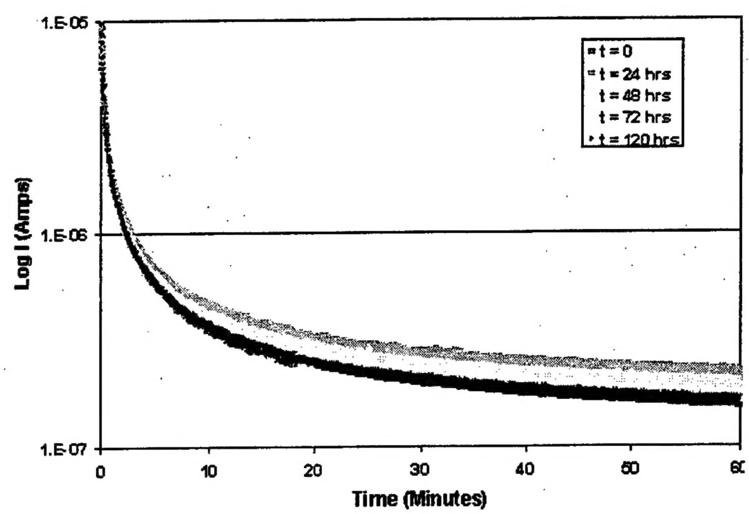

Figure 10. Applied current responses for 350 mV potential holds (1 hr duration) of commercial probe exposed to natural pond water for 120 hrs.

a) b) c)

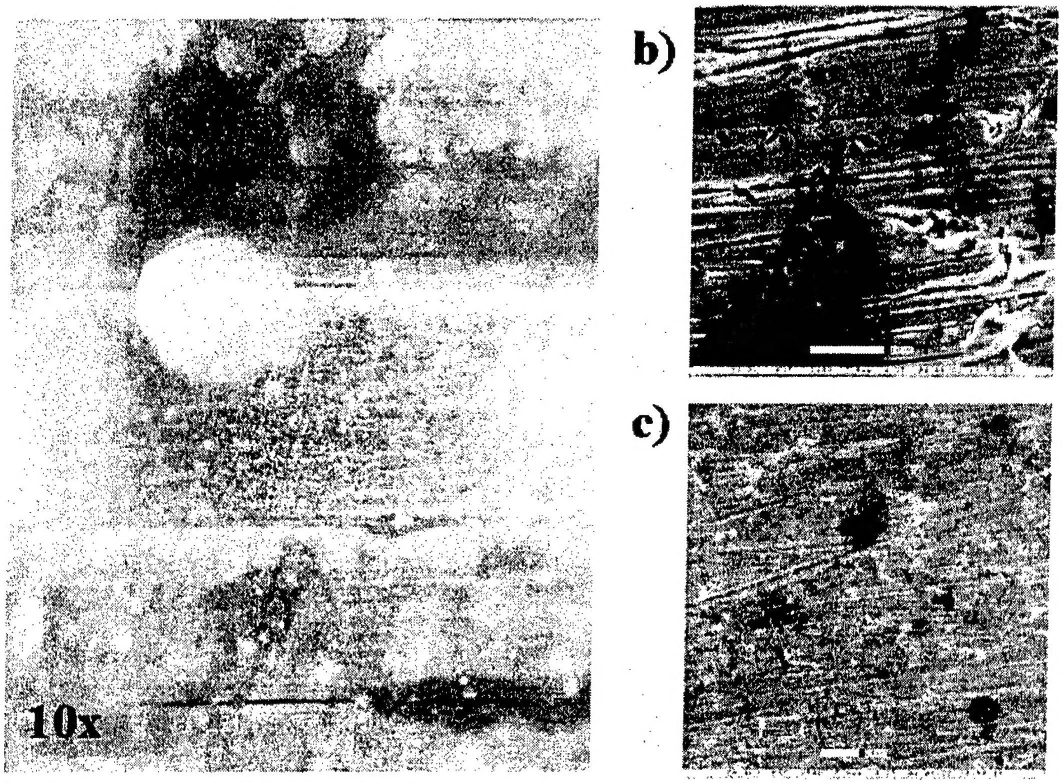

Figure 11. Positive electrode of commercial probe exposed to natural pond water for 120 hrs. a) Optical image of electrode surface after 120 hr exposure. b&c) Micrographs of electrode surface after 120 hr exposure.

a) b) c)

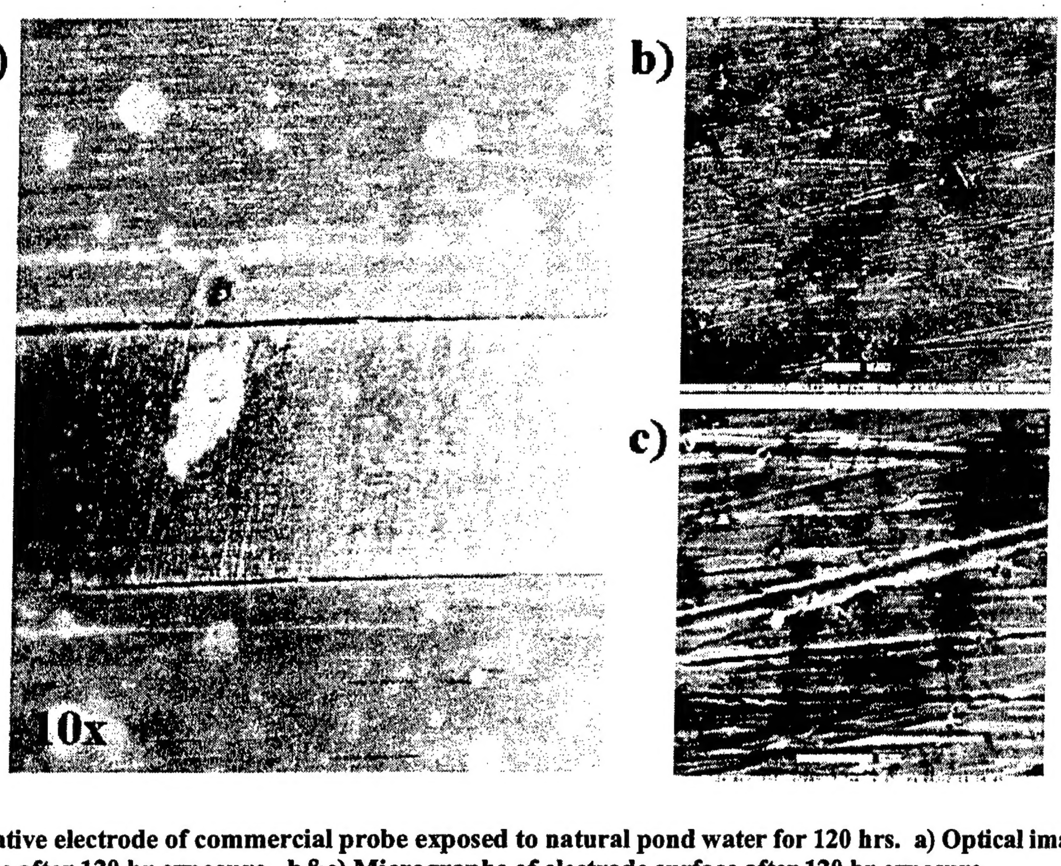

Figure 12. Negative electrode of commercial probe exposed to natural pond water for 120 hrs. a) Optical image of electrode surface after 120 hr exposure. b&c) Micrographs of electrode surface after 120 hr exposure.

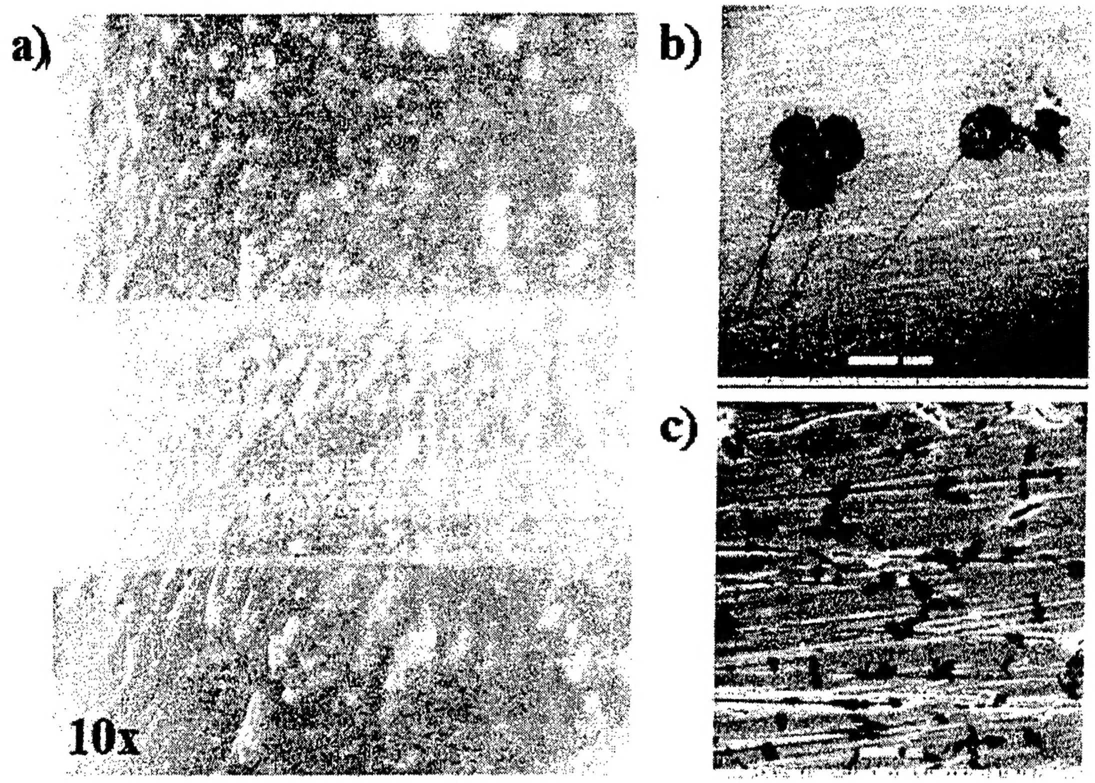

Figure 13. Commercial probe exposed to natural pond water for 120 hrs with no polarization. a) Optical image of electrode surface after 120 hr exposure. b&c) Micrographs of electrode surface after 120 hr exposure.

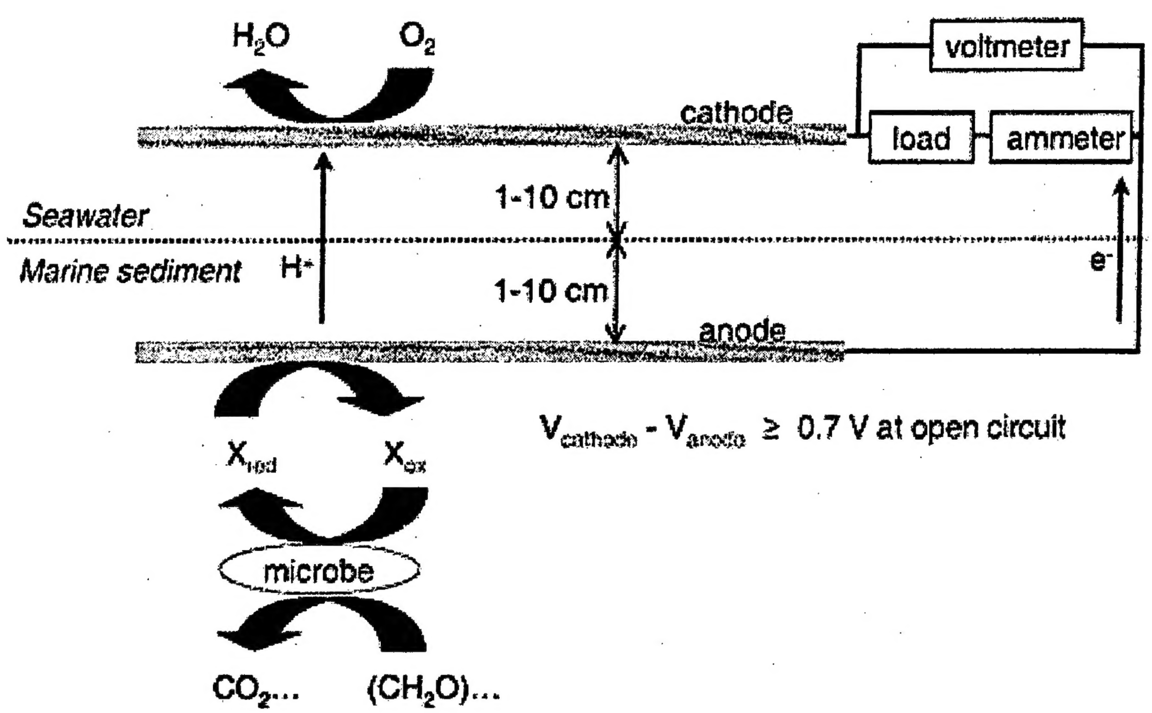

Figure 14. Schematic by Reimers *et al.*[20] illustrating the electrochemical processes of a microbiologically mediated battery.